

Original Research Article

**CHROMATOGRAPHY AND MASS SPECTROSCOPY ANALYSIS OF
BIOACTIVE PRINCIPLES FROM *VERNONIA AMYGDALINA* LEAF
AQUEOUS EXTRACT**Adiukwu PC^{1*} and MO Tebogo¹**Paul Chukwuemeka Adiukwu**

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ABSTRACT

Application of medicinal plants in managing disease conditions is a practice as old as mankind. Its use in today's healthcare has increased astronomically when compared to any other era. National policies, which integrate herbal products in healthcare systems, and the increasing presence of herbal clinics have become the order in many countries. Despite the ease of accessibility and affordability, the use of products from medicinal plants as phyto-medicines is threatened by the inability to maximize the benefits. This is due to inadequate qualitative and quantitative data necessary for proper application and regulation. *Vernonia amygdalina*, a herb widely used by ethnics in diverse forms of health management, is one such medicinal plant. This study was designed to determine referenceable values for the ethno formulation of the herb which is usually prepared as the aqueous extract of the leaf. Standard techniques and procedures were employed for this study. Fractionation of the extract was carried out using facilitated column chromatography. Pure principles of fractionates were separated with gas chromatography and identified using hyphenated mass spectrometer based on their relative abundance. The obtained chromatogram and spectra of principles were elucidated by relating data to the Mass Spectral Database with Automatic Mass Spectra Deconvolution & Identification System (AMDIS). Preliminary screening of extract indicated the absence of quinine but presence of alkaloids, tannins and saponins. Aqueous extraction produced 18 % (w/w) yield. The accelerated column chromatography produced a yield in the ratio of four to six to nine for the chloroform, chloroform/methanol and methanol effluents, respectively. Data obtained from the AMDIS elucidation showed the presence of eleven principles, which includes 1, 2, 3, 4-Butanetetrol; 1, 2-Benzenediol; and Caprolactam among others. Some of the properties and bioactivities of these principles have been reported in previous literature. Findings suggest that bioactivity common with some of these principles is consistent with previous literature on the use of the herb, and demonstrates reasons for the folkloric application.

Key words: Chromatogram, flash column, extract, hyphenated technique, leaf, medicinal, spectra



INTRODUCTION

The contemporary use of herbal products has transcended the traditional nutritive concept. Its application in phyto-medicines dates back to the Paleolithic age [1]. Today, herbal formulations in commerce have been diversified into various areas of application: dermatological, dietary replacement, insecticide, pesticide, and medicinal products. At least 80% of the World Health Organization (WHO) member states utilize vocations like the Traditional and Complementary Medicine where such products are used. Putatively, 88% of these member states acknowledge the use of traditional medicine in their states [2]. The global market for herbal products was estimated at \$83 billion [3]. These products are considered to be of primary healthcare importance to approximately 85% of the world's populace [4]. India exported \$330.18 million worth of herbal produce during 2017-18 [5]. Prior to this period, India's herbal related commerce was estimated at \$10 billion while the annual herbal drug production in China was \$48 billion [6, 7]. In developing countries, 65 to 80% of the populace use medicinal plants as remedies [3]. Available information shows that the estimated number of adults who ever used herbal preparations and dietary supplements in the USA increased from 50.6 million in 2002 to 53.6 million in 2012 [8]. The report of the China State Council Information Office in 2016, shows that trade in traditional Chinese medicine has maintained a rapid growth [9].

The use and application of phyto-medicines is on the increase. Current data suggest that affordability and accessibility have become comparatively better for herbal than allopathic medicinal products [10]. Most of the uses and application techniques of the herbal medicinal products are considered to emanate from indigenous activities, which connotes cultural acceptability among ethnics. In an effort to exploit the associated medicinal benefits and popularity of such products among ethnics, various countries are increasingly adopting phyto-medicines into their national alternative healthcare policy [10]. Records have shown a surge in the increasing number of herbal clinics as compared to any other era in modern day healthcare system [2].

Vernonia amygdalina (Asteraceae) is a herbal medicinal plant grown in a range of ecological regions in Africa and the Arabian Peninsula. It is a 2 – 5 m high shrub with 6 mm diameter petiolate and elliptic shaped leaf. The leaves are green with characteristic odor and a bitter taste. It has familiar local names: “moquene” in Setswana, “awonoo” (Southern Ghana), “omubirizi” or “omululuza” (West and Central Uganda), “olusia” (Luo, Kenya), and “ewuro”, “etidot” and “olugbo” (Southern Nigeria) [11, 12]. The leaf is referred to as “bitter leaf” because of the bitter taste. The use of this plant among ethnics is basically folkloric. As a nutritive delicacy, processing of the leaf for human consumption involves soaking and washing in warm water to remove the bitter tasting extract. This extract is considered medicinal and perhaps, responsible for the health benefits [13]. The leaf is commonly macerated by ethnics for medicinal purposes, and the obtained aqueous extract orally administered in an unstandardized manner. Among the proffered reasons for the ethno-administration include malaria fever, pains, anthelmintic, febrile convulsion, stomach infection, et cetera [14, 15].



Vernonia amygdalina is a well-researched herb in respect of the medicinal and nutritive benefits. The practical non-toxic nature of the herb has been known over centuries of its use as a delicacy by ethnics. Usually administered orally for medicinal effect, the saponin content is considered hydrolyzed by the enzymatic systems in the alimentary canal. The constituting phytochemical profile of the herb has become common knowledge among scholars. Recent findings showed the presence of novel compounds [16, 17]. However, observed activities of the herb may not necessarily exclude the presence of known principles that may have been identified in other sources.

A challenging factor in quality control of herbal products is the complexity of the constituting phyto-principles which are, in most cases, not clearly elucidated. Because of the limited information on the structure and properties of these compounds, setting specifications and standards for the products is usually an uphill task. Unfortunately, the use of unstandardized herbal products like *Vernonia amygdalina* ethno formulation in phyto-medicine confronts the same outcome as may be common with adulteration [3, 18]. The highlight of the associated limitations to the use of this formulation is premised on the regulatory and quality control challenges [10]. Presently, there is a lacuna in the available quantitative and qualitative index for the rational use and regulation of the herbal products from *Vernonia amygdalina*. One of the acceptable approaches to address quality amidst this complex nature of the herb is the provision of a finger print pattern that can be unique for the product. Such a parameter can be generated by using the gas chromatography and mass spectroscopy (GC-MS) hyphenated technique which is useful in herbal identification and quality monitoring of products. Therefore, the aim of this study was to investigate the chromatography and mass spectroscopy data that can be used in advocating for policies for products from the aqueous extract of *Vernonia amygdalina* leaf.

MATERIALS AND METHODS

Chemicals, Drugs and Test Agents

Analytical grades of methanol, n-butanol, diethyl ether, chloroform, acetone, xylene and methanol (chromatography grade) were obtained from British Drug House (BDH) sales representative in Kampala, Uganda.

Plant Material and Extraction

The fresh leaves of *Vernonia amygdalina* identified by a botanist were collected in the morning, between June and July in the south-western region of Uganda. Specimens were retained in the Pharmacy Department, Faculty of Medicine, Mbarara University of Science and Technology. The leaves were shade air-dried and ground into a coarse powder that was passed through a sieve (15:460) of mesh size Bs: 16 to obtain 2000 g fine powder. Following the procedure as described in previous literature, the moistened powder was allowed to stand for 15 minutes before maceration for three hours in warm (<80° C) distilled water at a ratio of 131 g to 9.0 liters [19]. Intermittent shaking was employed during the maceration. Using a filter paper, the filtrate was collected while warm, and filtered further using a Buckner assemblage (aided by a suction pump). The final filtrate was subsequently evaporated to dryness in an oven at ≤ 80° C. The total



yield of 360 g (18 %) was obtained and the dried powder stored in a desiccator at 25°C for further use.

Phytochemical Screening

Preliminary screening of the aqueous extract for phytochemicals was carried out by wet chemical reactions using standard procedures [20]. A 10 ml solution of 1 mg/ml of the dried extract was prepared. Using 1 ml each, basic tests were carried out to identify the presence or absence of saponins, alkaloids, terpenoids, sophisticated lactones, triterpenoids, reducing sugars, amino acids, tannins, cardiotoxic glycosides, quinin and flavonoids. Used in the evaluation are foaming, Dragendorff's, Salkowski, Baljet, Liebermann-Burchard, Fehling, Ninhydrine, ferric chloride, Kedde's, Borntragers tests and Shinoda assays, respectively.

Accelerated Column Chromatography of Extract

Following the procedure as previously described by Adiukwu *et al.* [12], the extract was adsorbed onto a thin layer chromatography (TLC) grade silica gel (CSI 010, Unilab) at a ratio of two to five and dried at $\leq 80^{\circ}\text{C}$ to produce a free-flowing powder. Thirty milligrams of this powder was loaded and fractionated on a silica gel (May & Baker Dagenham, England: 0.2-0.5 mm, pore size 40 angstrom, 30-70 mesh) containing column. The column was eluted in multiples of 100 ml with gradient mobile phases of increasing polarity starting with chloroform; combination of chloroform and methanol (at a ratio of one to one); and methanol. Elution was facilitated using an air pump (Merck, Germany). Each 100 ml effluent was profiled using Thin Layer Chromatography (TLC) with a mobile phase system of acetone, chloroform and methanol at a ratio of 1:4:2. With the saturated iodine chamber, spots were located on the TLC plate and effluents with similar profiles were bulked together, concentrated and allowed to evaporate to dryness at room temperature. This resulted in 3 fractionates: 0.85 g (chloroform effluent), 1.35g (chloroform/methanol effluent) and 1.93 g (methanol effluent). Effluent concentrates were air dried and preserved for subsequent evaluations.

Gas Chromatography (GC) and Mass Spectroscopy (MS) Evaluation of Fractionates

Sample preparation: The dried chromatography effluent of the aqueous extract was dissolved in a test tube using excess methanol. The resulting solution was sonicated and filtered via the septum into the injector, which was maintained at the oven initial temperature of 40°C.

Mobile Phase: Helium carrier gas maintained at a constant flow rate of 14.8 ml/min was used as the vehicle.

Stationary Phase: Capillary column (DB5) of 30 m long, 250 μm in diameter and a film thickness of 0.25 μm was used as the stationary phase.

Chromatogram development: Following the standard procedure, 10 μl of sample as prepared above was introduced into the GC (Shimadzu 2010) set at an inlet temperature of 100° C. The oven temperature held at 40° C for 1 minute was gradually increased



with a rate of 10°C per minute to 290°C. Total run time for each sample was 25 minutes at a constant flow rate of 14.8 ml/min. The flow was split before introduction into the MS detector.

Mass Spectra Development: MS detector (Shimadzu QP2010) with EM voltage of a scan mode Electron Impact (EI)) at 70 eVolts was used. The lower and upper mass limits was set at 40 and 550 m/z. Quadrupole temperature and ion source temperature was 200°C. Spectra were obtained using Labsolutions software provided by Shimadzu GC-MS solution. Obtained spectra were compared to the spectra in the NIST/EPA/NIH Mass Spectral Database with Automatic Mass Spectra Deconvolution & Identification System (AMDIS) Program. The library search was performed with the NIST Mass Spectra Search program. Obtained spectra were documented as results. The same procedure was followed for the three different fractionates.

Analysis

Spectra data analysis were based on the NIST/EPA/NIH Mass Spectral Database with AMDIS Program. Interpretation of identified compound was considered as the relative abundance of the m/z ratio indicated by the spectra peaks (Mass vs Relative Intensity).

RESULTS AND DISCUSSION

Pooling of data to vet authenticity in a correlational manner to the usability of a medicinal product can be a key factor in products' regulation. The study was able to qualitatively evaluate the aqueous extract of *Vernonia amygdalina* leaf based on the ethno formulation.

Aqueous extraction: Ethnics, particularly in Africa, are known to formulate herbal medicines using aqueous or locally fermented alcohol as a vehicle. Although, the alcoholic medium may provide an inhibitory and preservative activity against microbial growth, many of these preparations are often formulated using aqueous base. The reason may be because of the ease of accessibility and affordability of potable water. A limiting factor with the use of aqueous vehicle is the propensity to also dissolve inorganic principles that may not necessarily be part of the plant material. The tendency to dissolve environmental inorganic materials or soil associated with the herb suggests why wide variability may be observed for aqueous extractive values.

Phytochemicals are mostly organic in nature. A good number of them are expected to exhibit significant solubility in known organic solvents. However, some of these principles are known to also show minimal to sufficient solubility in aqueous medium. Such characteristic may reflect the physical expression of the different moieties associated with the structure. In accordance with this concept, the use of aqueous extraction in this study, in line with the ethno-formulation, seeks to isolate phyto-principles that may exhibit minimal to sufficient solubility in aqueous medium. In this regard, it may be appropriate to consider some of these principles, as contained in the extract, to be responsible for the observed health benefits of the ethno-formulation.



Accelerated column chromatography: Principles that dissolves freely in polar solvent like water are commonly considered to be polar in nature. Also, some organic solvents like alcohol tend to provide a similar solubilizing medium for some of these polar compounds. Utilization of the interplay of polar and non-polar mobile systems, such as methanol and chloroform, to achieve gradient elution in chromatography is premised on solubility of compounds in these solvents. Basically, it shows the relative interaction of the compounds with the solvents and ultimately, their retention times in such mobile phases. Such interaction suggests the degree of polarity of the different compounds. Application of gradient elution in this study provides an insight into the relative degree of polar characteristics of the respective compounds contained in the herbal extract.

Phytochemical: Results from the preliminary phytochemical screening reveal the possible presence of saponins, alkaloids, terpenoids, sophisticated lactones, triterpenoids, reducing sugars, amino acids, flavonoids, tannins and cardiotoxic glycosides. This is consistent with previous reports [12, 21]. Further phytochemical details were obtained with the AMDIS program, which showed the presence of eleven known compounds as contained in Table 1. The compounds were identified using their name, empirical (and structural) formula, as well as, their molecular weights.

GC Chromatogram and MS Spectra: The GC chromatogram for the chloroform effluents (Figure 1) indicates the presence of four principles. Among these principles is 1, 2, 3, 4-Butanetetrol (Figure 4), which has been noted for its anti-inflammatory effects due to the ability to inhibit cyclooxygenase enzyme [20].

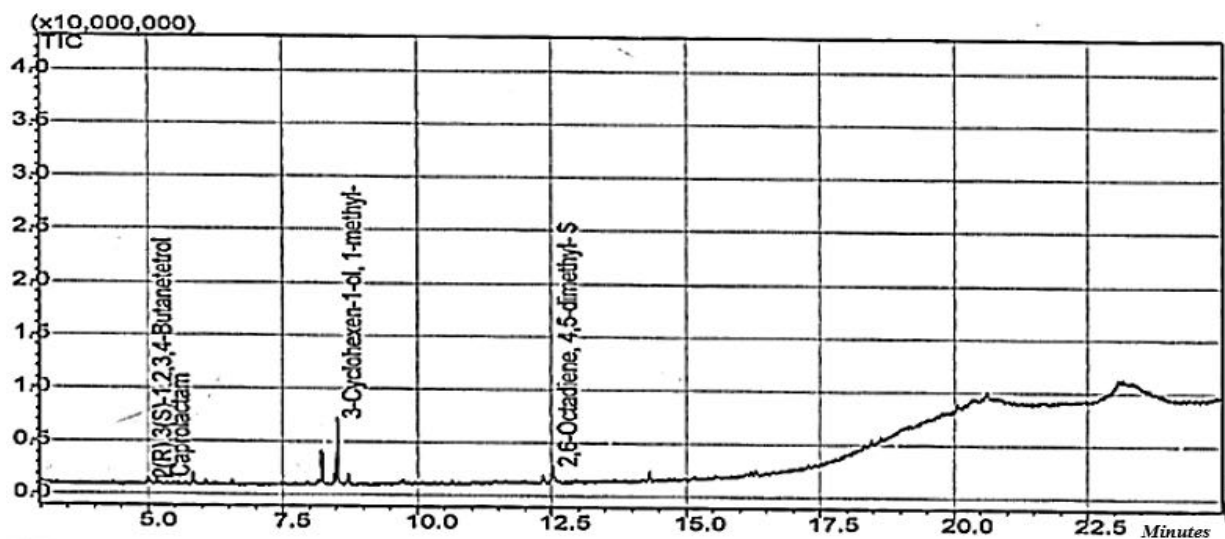


Figure 1: Chromatogram of compounds contained in chloroform fraction

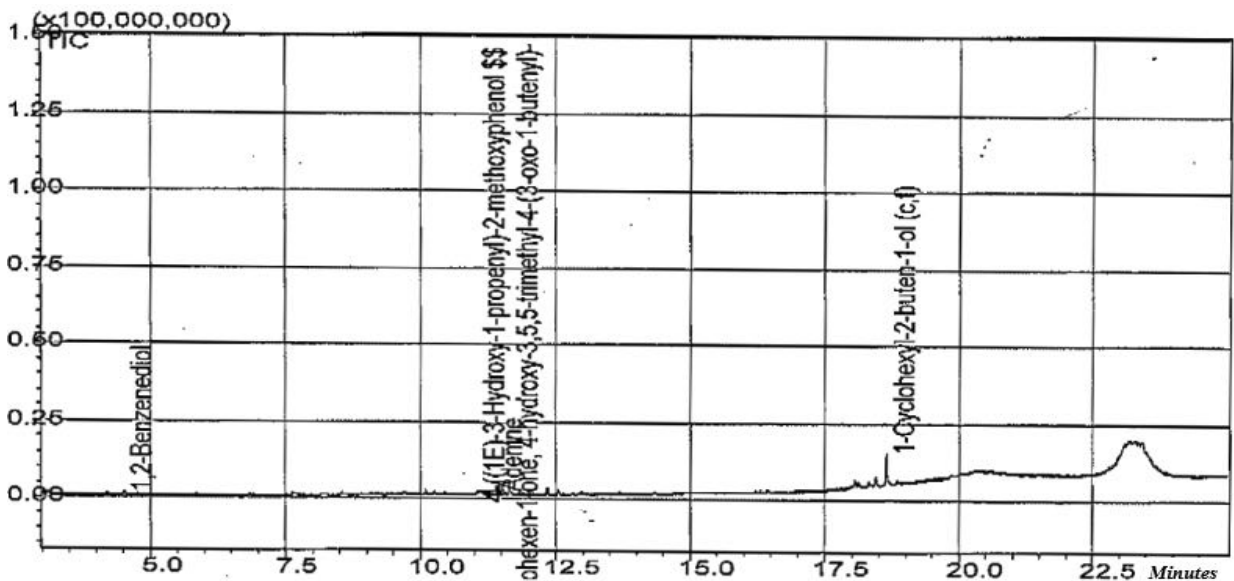


Figure 2: Chromatogram of compounds contained in chloroform/methanol fraction

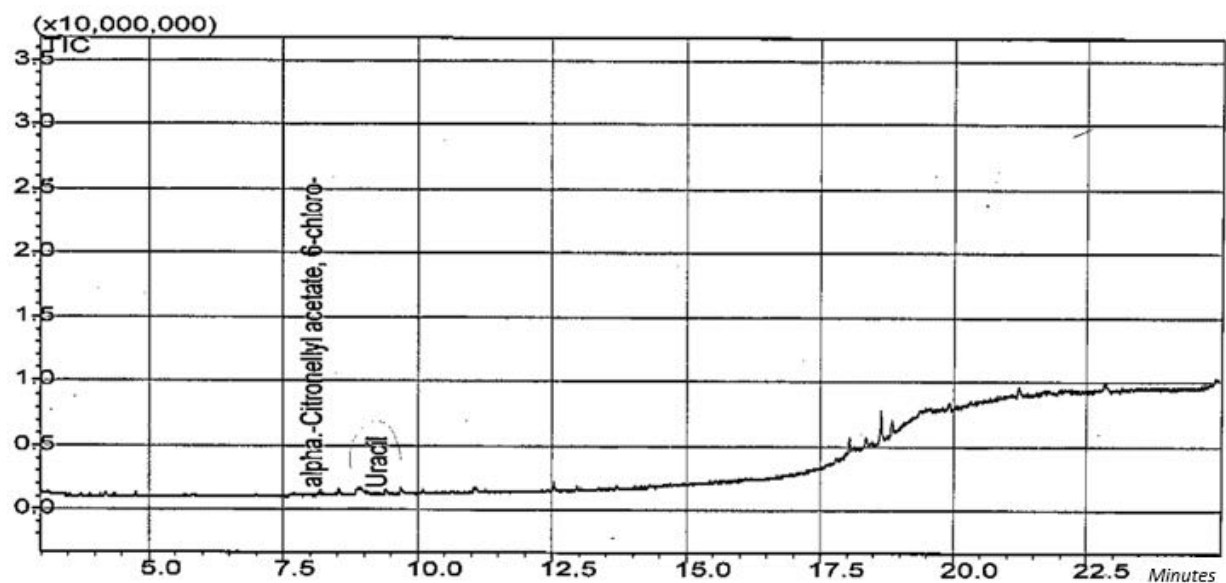


Figure 3: Chromatogram of compounds contained in methanol fraction

Butanetetrol, a sugar alcohol (polyol), is commonly called erythritol and has been reported to possess coronary vasodilation and anti-nociceptive properties. Prior animal study showed that *V. amygdalina* extract has anti-inflammatory, antipyretic and antinociceptive properties [12, 22]. Available scholarly data have also shown the benefits of the herbal extract in hypertension [23, 24]. Perhaps, the presence of this principle may explain why ethnics use the herbal extract in the treatment of cardiac diseases and fever occasioned with aches and pains.

Paradoxically, ethnics have reported a sweet after-taste with meals prepared using the leaf of *V. amygdalina*. This has been recognized in some scholarly reports, especially as related to the chewing of the stem [25]. Butanetetrol is an approved flavor enhancer

due to the sensory effect of its meso isomer [22, 26]. Though not sufficiently explained, 4-Hydroxy-3, 5, 5-trimethyl-4-(3-oxo-1-butyl)-2-cyclohexen-1-one, a compound isolated from the methanol/chloroform effluents (Figure 2) and identified in the MS spectra (Figure 8), has also been reported as one of the many recognized flavoring agents by the World Health Organization [27].

Caprolactam (Figure 5), a compound isolated from the chloroform effluents is considered a lactam of caproic acid. It is a precursor to nylon 6, a widely used synthetic (fiber) polymer. However, caprolactam in aqueous solution hydrolyzes to amino-caproic acid, which is a useful inhibitor of proteolytic enzymes like plasmin: a fibrinolytic agent [28]. For this reason, caprolactam is considered effective in the treatment of certain bleeding disorders and may strongly suggest why *V. amygdalina* herbal extract is used by ethnics to treat bleeding wound [29]. Also, the use of this herbal extract as antimicrobial may be occasioned by the presence of 1-methyl 3-cyclohexen-1-ol (Figure 6) [30]. A similar structural analogue, 3-cyclohexen-1-ol, which is a volatile oxygenated monoterpene found in the essential oil of *Hyptis suaveolens*, has been reported to be antimicrobial [31].

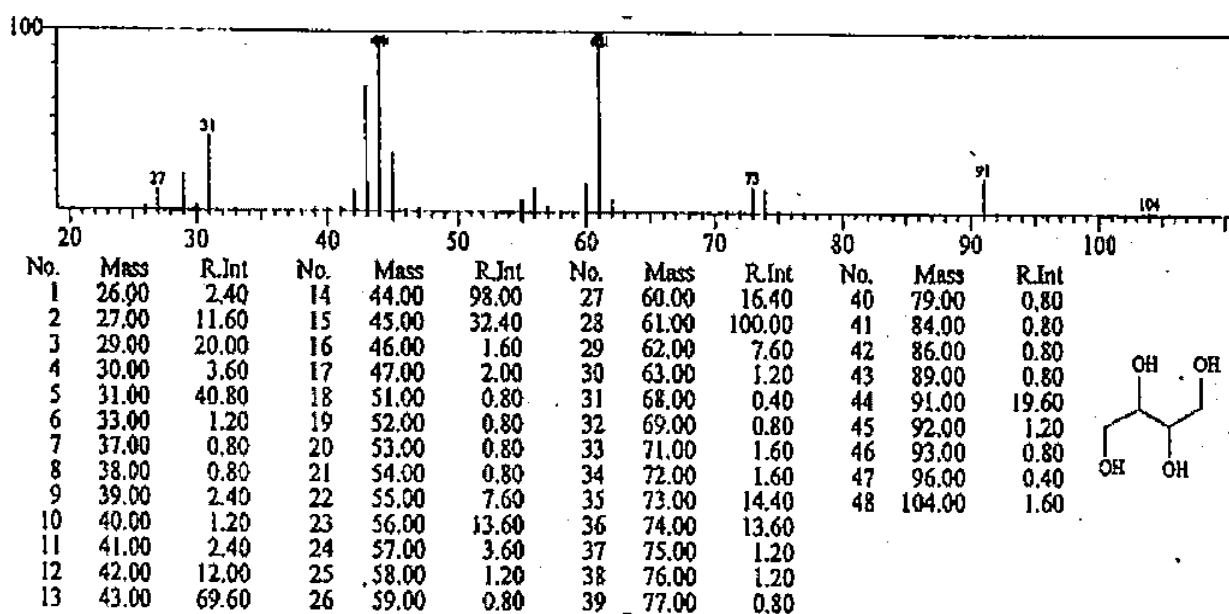


Figure 4: MS spectra of (2R, 3S)-1, 2, 3, 4-Butanetetrol present in the chloroform effluents

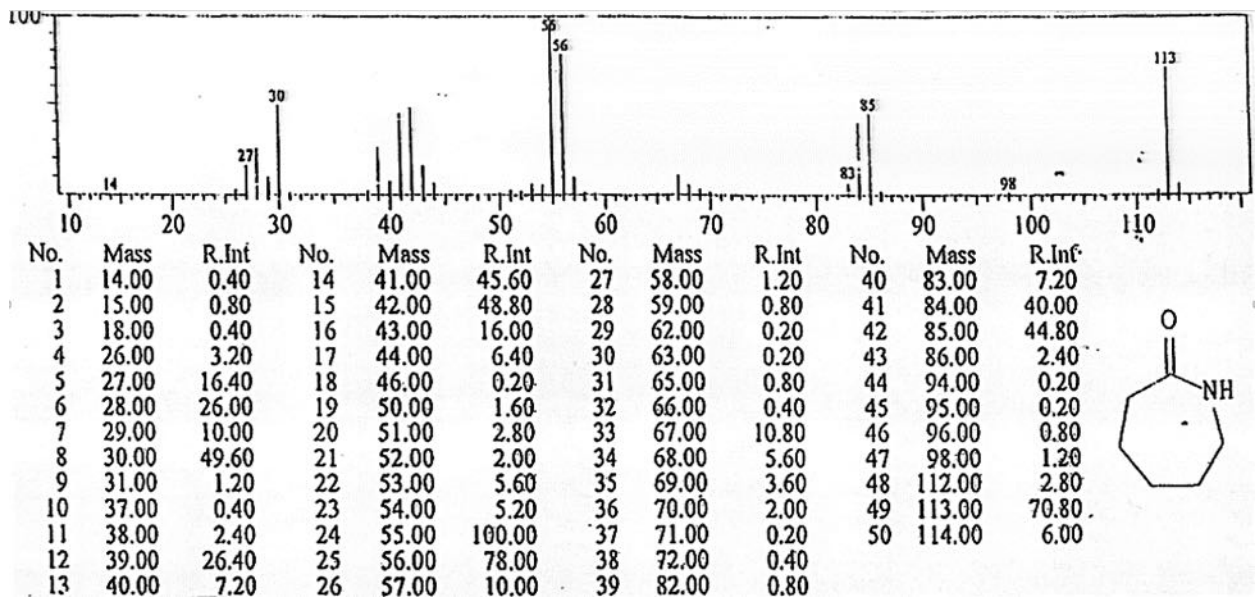


Figure 5: MS spectra of Caprolactam present in the chloroform effluents

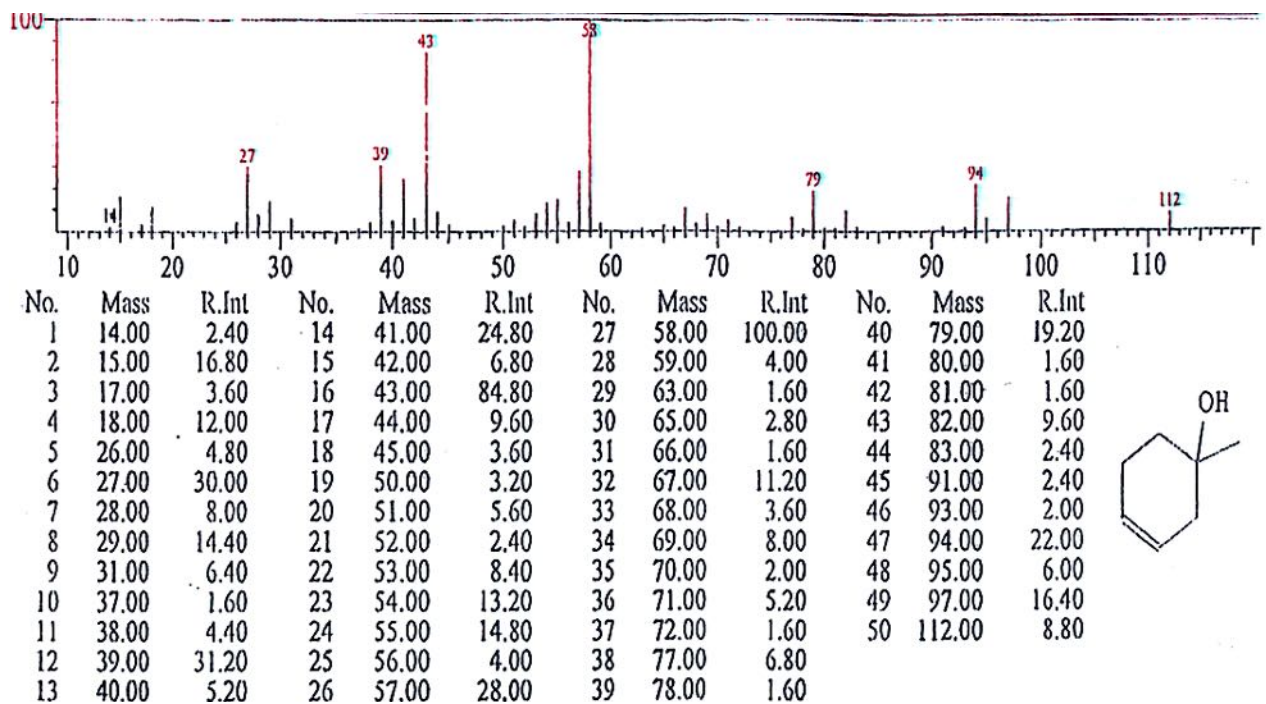


Figure 6: MS spectra of 1-Methyl-3-cyclohexen-1-ol present in the chloroform effluents

Another principle identified in this study is 1,2-benzenediol (Figure 7) in the chloroform/methanol fraction (Figure 2). Prior reports show that the compound is antineoplastic and also, a precursor of the antibacterial tetrachloro derivatives. This may explain the antioxidant and antineoplastic property observed with the herb [32, 33, 34]. The use of the herbal preparation in managing allergic episodes in traditional medication may be due to the presence of the coniferyl alcohol, 4((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol, which is one of the monolignols and has been reported to relieve allergic headache [35, 36]. 6-Chloro- α -Citronellyl acetate (Figure 3) is an ester

derivative of citronellol, a reduced form of citronellal. The presence of this aldehyde has been shown to be a major contributor of antifungal and antimicrobial effects [37].

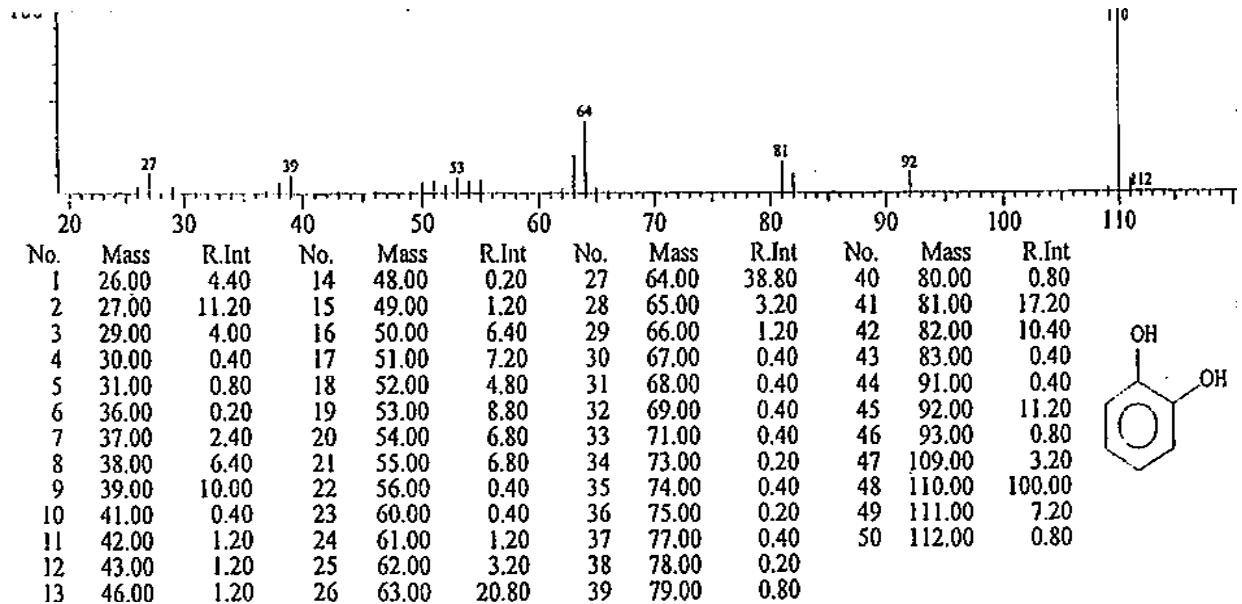


Figure 7: 1, 2-Benzenediol present in chloroform/methanol effluents

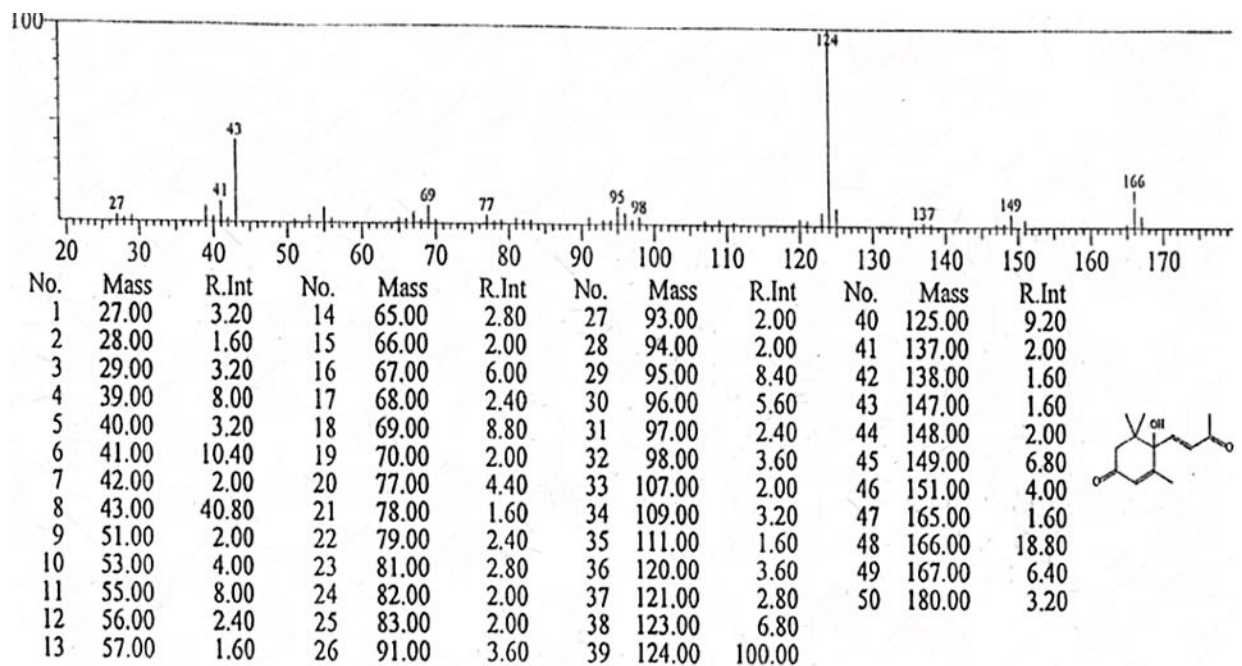


Figure 8: 4-Hydroxy-3, 5, 5-trimethyl-4-(3-oxo-1-butyl)-2-cyclohexen-1-one present in chloroform/methanol effluents

A compound identified in the chromatogram for chloroform effluent (Figure 1), 4, 5-dimethyl 2, 6-octadiene is an isomer of 2, 6-dimethyl 2, 6-octadiene, which is widely distributed in plants. It is a dimer of isoprene, a precursor of the spiroketal steroidal nucleus and pentacyclic triterpenoid structures. These structures have been shown to be present in previous studies of the *V. amygdalina* leaf extract [12, 16,]. The presence of

adenine and uracil, which are purine and pyrimidine bases may not be of any medicinal significance as they are widely distributed in plants and animals. They are known for the synthesis of ribonucleic and deoxyribonucleic acids. However, uracil has been identified in the synthesis of 5-fluorouracil, a compound known for its antineoplastic activity and effective in managing cancerous cell.

CONCLUSION

The study was able to show eleven phytochemical principles, which can be key to regulating and identifying products formulated using *V. amygdalina* aqueous extract. The presence of some of these principles further demonstrated the basis for some of the reported ethnic and trado-medical uses of the herbal preparation. Also, these principles can be useful in rationalizing dosage regimen for the ultimate therapeutic benefit of the herb. The finger-print impression of the GC-MS data from the study provides policy makers genuine information that will contribute to quality regulation of product. However, obtained data could not sufficiently establish the relative abundance of these compounds or elucidate on their effective dose, which rests the fulcrum for the ultimate therapeutic outcome in man. Further investigation of these compounds relative to the dosage regimen of the extract, will avail more information that maybe beneficial to the application of the herbal product in the health and research sectors.

ACKNOWLEDGEMENTS

Gratitude goes to the staff and management of Natural Chemotherapeutic Research Laboratories and the Government Analytical Laboratory, Kampala Uganda for their technical support.

COMPETING INTERESTS/AUTHORS' DISCLOSURE STATEMENT

Authors declare that no competing financial interests exist in this study.



Table 1: Formula and molecular weight of phyto-principles identified using the Automatic Mass Spectra Deconvolution & Identification System

S/N	Column Fractionates	Name	Empiric Formula	Molecular weight
1	Chloroform	(2R, 3S)-1, 2, 3, 4-butanetetrool	C ₄ H ₁₀ O ₄	122
2		Caprolactam	C ₆ H ₁₁ NO	113
3		1-Methyl-3-cyclohexen-1-ol	C ₇ H ₁₂ O	122
4		4, 5-dimethyl 2, 6-octadiene	C ₁₀ H ₁₈	138
5	Chloroform/Methanol	1, 2-Benzenediol	C ₆ H ₆ O ₂	110
6		4-Hydroxy-3, 5, 5-trimethyl-4-(3-oxo-1-butyl)-2-cyclohexen-1-one	C ₁₃ H ₁₈ O ₃	222
7		4((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	C ₁₀ H ₁₂ O ₃	180
8		Adenine	C ₅ H ₅ N ₅	135
9	Methanol	1-Cyclohexyl-2-buten-1-ol (c, t)		
10		6-Chloro- α -citronellyl acetate	C ₁₂ H ₂₁ ClO ₂	232
11		Uracil	C ₄ H ₄ N ₂ O ₂	112

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